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Plaque/Focus Immunoassay: a Simple Method for Detecting Antiviral Monoclonal or Other Antibodies and Viral Antigens in Cells

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A new, simple enzyme-linked immunosorbent assay (ELISA) is described which is performed directly on infected and fixed cell cultures in microtitre plates. It permits large scale screening of antiviral monoclonal antibodies and differentiation of specific antibodies from those usually responsible for high background reactions in other ELISA techniques. Time consuming purification of antigens is thus avoided.

The plaque/focus immunoassay is also applicable to titration of antibodies in patients' sera and antigens in lytically or non-lytically virus-infected cells. It may also be used to localize antigens in different cell compartments. This immunoassay requires no special equipment and results may be evaluated either with the naked eye or using a light microscope.

Key words: *enzyme immunoassay – monoclonal antibody – viral antigens – virus-specific antibodies*

Introduction

Enzyme immunoassays are convenient and sensitive for tracing antibodies during production of monoclonal antibodies, when large scale screening of small amounts of supernatants, possibly with low antibody titres, is required. Experience shows that conventional ELISA techniques result in selection of clones producing antibodies against serum proteins or cell fractions even when gradient-purified virus preparations are used as antigens. For this reason it is often necessary to select antiviral monoclonal antibodies by fluorescent antibody methods.

The need for a more convenient test system led to our modification of the usual ELISA. We have established a cell-bound immunoassay for the detection of virus-specific antigens or the corresponding antibodies. Uninfected cells surrounding plaques or foci of infected cells serve as internal controls, making tedious virus purification steps unnecessary. The most prominent advantage of this test is in screening of monoclonal antibodies, but the same immunoassay has been applied to many other virological investigations. It allows a quick determination of antibodies

in patients' sera as well as detection of antigens in lytically or non-lytically virus-infected cells.

Materials and Methods

Preparation of test plates

Trypsinized cultured cells were resuspended in growth medium (Eagle's medium Dulbecco's modification, EDM) supplemented with 2% fetal calf serum (FCS) and infected in suspension before plating into 96-well microtitre plates. For each plate about 2×10^6 cells were infected with $2-5 \times 10^3$ infectious units (focus or plaque forming units) in a total volume of 10 ml, taking 100 μ l suspension per well to give approximately 20-50 infectious units. The cultures were incubated at 37°C in a humidified incubator with 5% CO₂ until optimal antigen expression was achieved. Cultures infected with cytolytic viruses were in most cases fixed when changes in cell morphology became visible. For viruses which induce less pronounced or no cytopathogenic effect, the optimal incubation time was determined in preliminary tests.

Fixation

Fixation with mixtures of acetone and alcohol. The following fixative mixtures were prepared and used successfully: (a) 66% acetone, 34% methanol; (b) 66% acetone, 34% methanol, 0.75% formaldehyde; (c) 60% acetone, 25% ethanol, 15% methanol.

After removal of the growth medium, wells were washed once with the desired fixative solution. The plates were filled again with fresh fixative (200 μ l/well) and kept at -20°C (for at least 2 h). The plates could be stored for several weeks at -20°C. To avoid evaporation of the fixative, plates were kept in air-tight plastic bags.

Addition of fixatives, and all washings, were carried out with a self-filling syringe fitted with a continuous pipetting device connected to a multidispenser.

Fixation with formaldehyde or glutaraldehyde. Cells were fixed with 3% formaldehyde or 0.25% glutaraldehyde in PBS. To each well (still containing growth medium), 200 μ l of fixative was added and the plates were incubated at room temperature (RT) for approximately 10 min. The fluid was then poured off and new fixative solution (200 μ l/well) added. If drying was avoided, the plates could be stored for several months at 4°C.

For determination of intracellular antigens after aldehyde fixation, the plates were washed once with 1% Triton X-100 in PBS and then incubated with 200 μ l/well of the detergent solution for 30 min at RT.

Testing for antigens or antibodies

Before the immune reaction step, the fixative solution or Triton buffer was flicked off. The plates were washed once with a washing buffer, containing 1% FCS in PBS. This PBS/FCS solution (200 μ l/well) was also used for preadsorption, incubating

the plates for 1 h at RT. After removing PBS/FCS, 25–50 μ l/well of hybridoma culture supernatants from 96-well microtitre plates or test serum dilutions — also prepared in microtitre plates or test serum dilutions — also prepared in microtitre plates — were transferred to the test plates with a multichannel pipette and the plates incubated for 60 min at RT. The plates were washed 3 times with PBS/FCS and 25 μ l of appropriately diluted anti-IgG antibodies conjugated to horseradish peroxidase were added to each well. The secondary antibodies as well as primary antibodies were diluted in washing buffer. The various commercial anti-IgG antibodies used were all at 1 : 1000 dilution.

After a further incubation period (60 min, RT), the plates were washed 5 times and 50 μ l/well freshly prepared substrate solution was added. Substrate solution was prepared by dissolving 10 mg 3-amino-9-ethyl-carbazole (Sigma) in 3 ml dimethylsulphoxide and adding it to 50 ml 20 mM sodium acetate buffer (adjusted to pH 5.0–5.5 with NaOH) containing 400 μ l 3% H₂O₂.

Thirty minutes after addition of substrate solution and before evaluation, the reaction was stopped by washing the plates with tap water. Staining solution was inactivated with 1% sodium hypochloride.

Results and Discussion

Screening of monoclonal antibodies

The immunoassay described above was initially established for screening hybridoma cell cultures prepared against bovine herpes mammillitis virus (bovine herpesvirus 2, BHV-2). We initially selected such monoclonal antibodies after testing the supernatants in a conventional ELISA with gradient-purified virus as antigen. In the event, several clones, giving strong positive reactions, proved to be producing different kinds of undesired antibodies of irrelevant specificities. Similar observations have been made in other laboratories (Engels, Misra, personal communications).

The method described here does not prevent background reactions, but it shows clearly whether antibodies bind to infected cells (identifiable by rounding or fusion in the environment of plaques) or to uninfected antigen-free cells growing in the same or other wells and serving as internal controls. Fig. 1 illustrates this clearcut difference. Infected cells appear as large polykaryocytes with dark brown or reddish brown staining, while uninfected cells between the plaques remain unstained or pale brown. Examination under low power microscopy differentiates between antibodies directed against intranuclear antigens, e.g. naked herpesvirus capsids (Fig. 2A) and antibodies directed against intracytoplasmic antigens (Fig. 2B). We were interested in the detection of antibodies against strong immunogenic glycoproteins which are also exposed on the surface membrane of herpesvirus-infected cells (Glorioso and Smith, 1977; Pauli and Ludwig, 1977; Norrild et al., 1978; Bauke and Spear, 1979). For this purpose we chose glutaraldehyde fixation which does not damage cell membranes (Fig. 3). Comparison of the fixation methods in respect of availability or preservation of intracellular antigenic determinants in this virus cell system showed

that most of the monoclonal antibodies gave the best reaction with formaldehyde-fixed antigens. Glutaraldehyde fixation gave comparable results, but the staining was less intense. Acetone/methanol-fixed antigens were detected poorly by some of the monoclonal antibodies. Only one of approximately 60 different monoclonal antibodies tested reacted better with acetone/methanol than with aldehyde-fixed antigens. The three different acetone/alcohol fixatives gave comparable results. For BHV-2-infected cells we preferred solution (b) because it slightly diminished background reaction. Since only small volumes ($25 \mu\text{l}$) of culture supernatant are required, 2 or 3 plates treated with different fixatives may be tested in parallel, even while the hybridomas are still growing in microtitre plates. Early determination of monoclonal antibody class may be carried out with class-specific secondary antibodies.

Extension of the technique to other serological or virological questions shows the wide field of application of this test. Apart from those described here, several other viruses were investigated: viz. measles, herpes simplex types 1 and 2, pseudorabies virus, feline herpesvirus 1, feline calici-virus and porcine corona virus (TGE-virus).

The reliability of our plaque immunoassay for routine diagnosis of antibodies against bovine herpes virus 1 (BHV-1) in cattle was investigated in comparison with neutralization tests. Representative results are given in Table I. The two tests gave comparable results. Some non-neutralizing sera were clearly positive in the immunoassay, demonstrating the higher sensitivity of the ELISA technique.

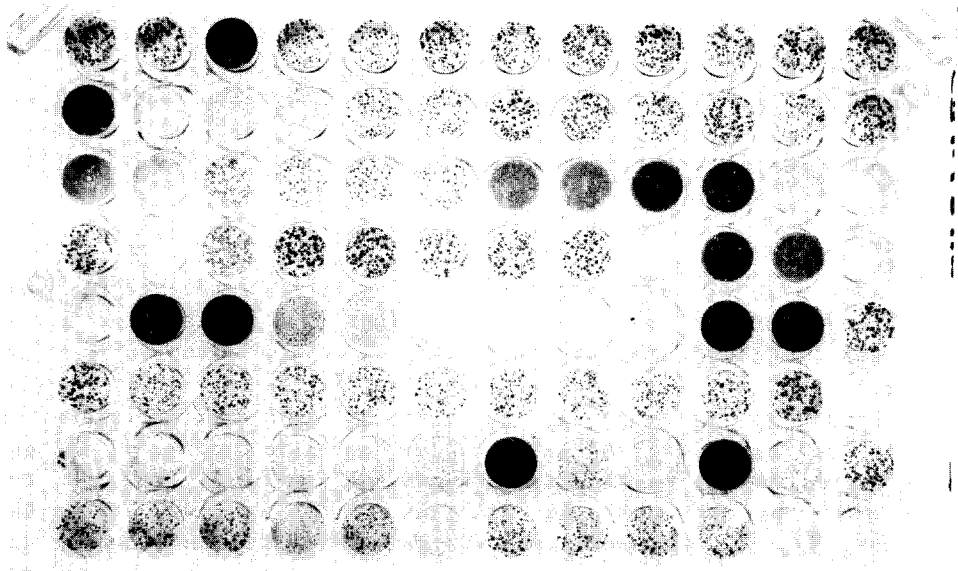


Fig. 1. Focus immunoassay on BHV-2-infected Georgia bovine kidney cells. Screening of monoclonal antibodies against BHV-2 on a microtitre plate. Positive, BHV-2-specific supernatants: foci of fused cells appear as dark dots; antibodies directed against uninfected cell antigens produce completely dark wells.

Detection of antigens or antibodies in non-cytolytic virus systems

Fluorescein-conjugated antibodies are of particular value for the detection of antigens or antibodies in non-lytic virus systems. Fluorescence assays (FA) in the

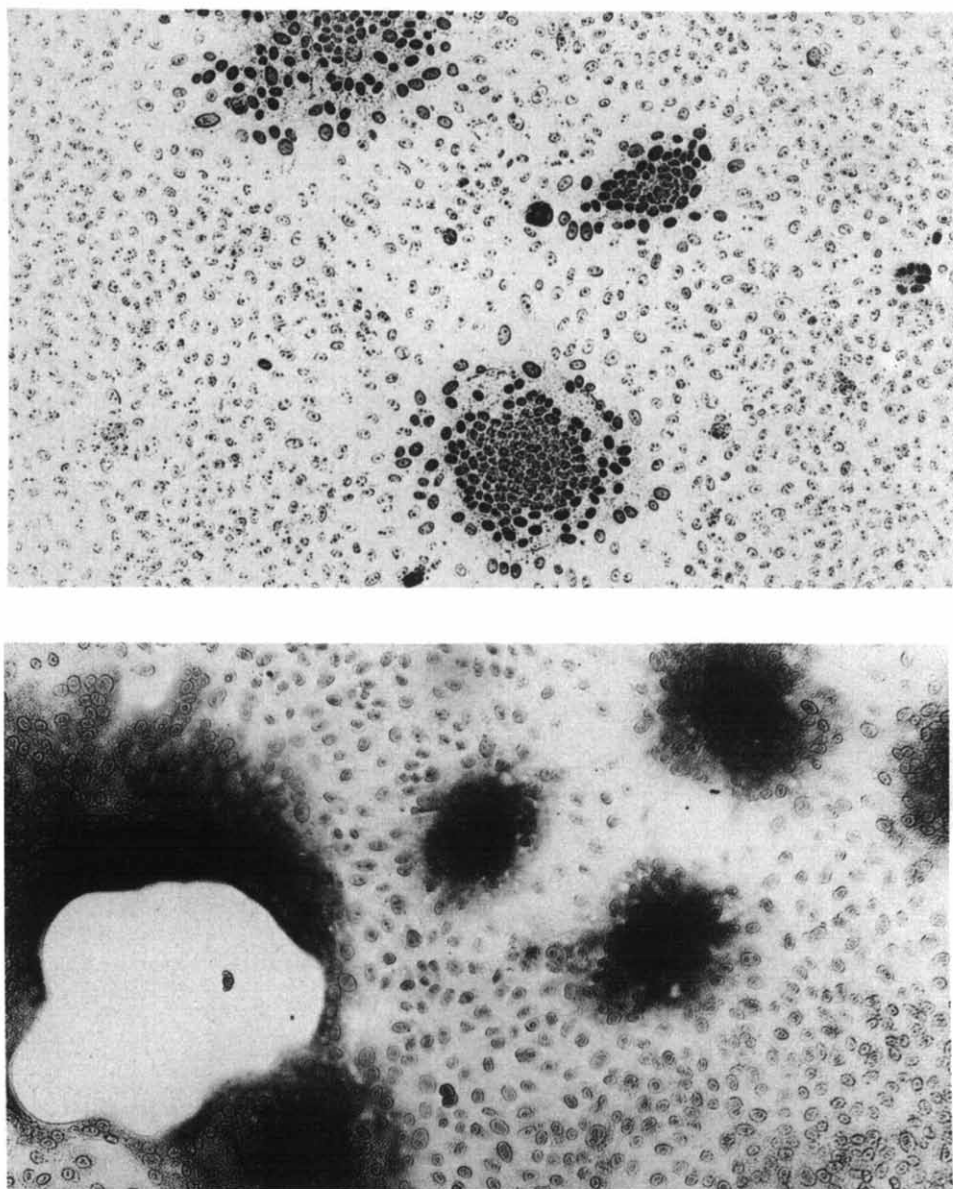


Fig. 2. Immunoassay after formaldehyde fixation and treatment with Triton X-100. A (top): monoclonal antibodies reacting mainly with viral antigens associated with the nucleus. B (bottom): same antibody as in Fig. 3, reacting also with antigens in the cytoplasm of infected cells.

TABLE I

COMPARISON OF NEUTRALIZATION TEST AND PLAQUE IMMUNOASSAY

Neutralization tests (NT) were performed as described by Gregersen (1983) in a constant virus-varying serum system, with 50 plaque-forming units for each well in a microtitre plate. Titres are given as serum dilutions causing 80% plaque reduction. ELISA was performed on BHV-1-infected cultures fixed with solution (b) and titres were macroscopically evaluated.

No. of sera	Titre	
	NT	Plaque immunoassay
8	<1:2	<1:20
1	<1:2	1:64
1	<1:2	1:512
1	1:4	1:64
1	1:4	1:128
1	1:6	1:128
1	1:6	1:512
1	1:10	1:128
1	1:10	1:256
1	1:12	1:256
1	1:300	1:2560
1	1:1410	1:10240
1	1:2790	1:5120

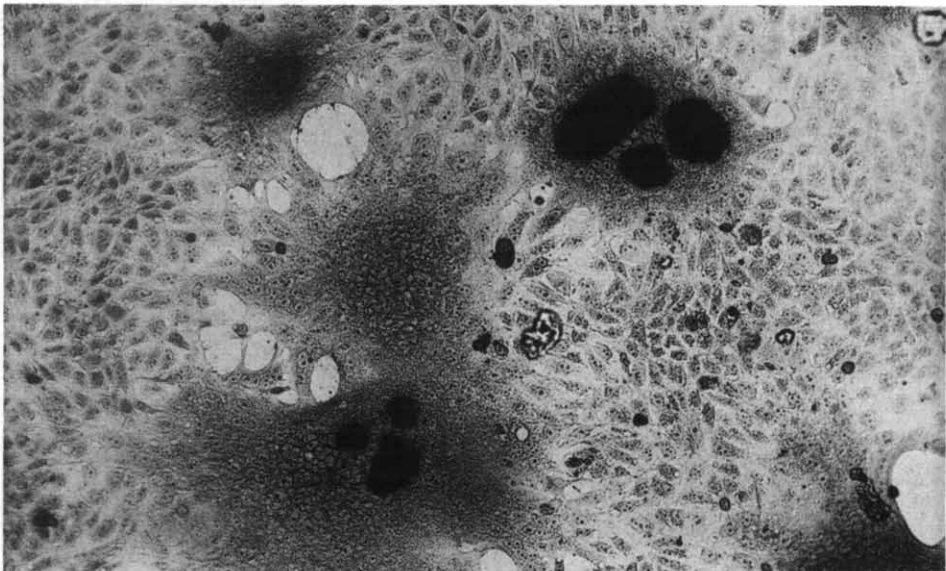


Fig. 3. Immunoassay after glutaraldehyde fixation of infected cells for detection of antibodies reacting with surface antigens of infected cells.

TABLE II

COMPARISON OF IMMUNOFLUORESCENCE (FA) AND FOCUS IMMUNOASSAY FOR DETECTING BORNA VIRUS-SPECIFIC ANTIGENS OR ANTIBODIES

Immunofluorescence and ELISA were performed in 96-well microtitre plates. Formaldehyde-fixed cells were treated with Triton X-100 before the immune reaction step. Some sera were also investigated by standard immunofluorescence on acetone-fixed cells (FA/standard; Hirano et al., 1983). Bound antibodies were detected with anti-immunoglobulin antibodies either fluorescein-conjugated (diluted 10^{-1}) or coupled to horseradish peroxidase (diluted 10^{-3}).

Serum	Species	Titre		
		FA	Focus immunoassay	FA/standard
1	Rabbit	1:2560	1:5120	1:80
2	Rabbit	1:320	1:1280	1:40
3	Rabbit	1:40	1:160	1:10
4	Rat	1:2560	1:2560	1:100
5	Rat	1:1280	1:5120	n.d.
6	Rat	1:640	1:10240	n.d.
7	Rat	1:320	1:1280	n.d.
8	Rat	1:80	1:160	1:10

n.d. = not done.

Borna virus system yielded important new information (Ludwig et al., 1973; Danner et al., 1978).

The focus immunoassay on Borna virus-infected cells provided tests for viral antigens as well as for antibodies without the taxing evaluation necessary when UV-light microscopy is used. Since it is known that fixation of antigens in virus-infected cells gives rise to particular problems, several fixatives were investigated and compared with standard acetone fixation used in the FA-technique. Formaldehyde fixation seemed to preserve Borna virus-specific antigens better than acetone/methanol or acetone. Most sera gave a lower background and higher titres on formaldehyde-fixed cells. Comparison of the FA and ELISA on formaldehyde-fixed cells showed that the ELISA was at least as sensitive as FA (Table II).

Acknowledgements

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